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Filed: May 6, 1998

REMARKS

Upon entry of the foregoing amendments, claims 7 and 9-12 are pending in the application. Claim 12 has been amended to correct errors in claim dependency. Because the foregoing changes introduce no new matter, their entry is respectfully requested.

Claim Rejections – 35 U.S.C. §103(a)

Claim 7 (and dependent claims 9-12) have been rejected by the Examiner under 35 U.S.C. §102(b) as being anticipated by *Nilsson, et al.* (Science 265:2085-2088, 1994) (*Nilsson*), but relies on U.S. Patent 5,162,109 (*Rajagopalan*), U.S. Patent 5,505,961 (*Shelley*), U.S. Patent 5,132,118 (*Mills*) and U.S. Patent 5,227,371 (*Rapaport*) to contend that the buffer of *Nilsson* is a pharmaceutically acceptable carrier. A proper 35 U.S.C. §102(b) rejection requires that a single reference expressly or inherently disclose each and every element of a claim. *In re Paulson*, 31 USPQ2d 1671, 1673 (Fed. Cir. 1994); MPEP § 2131 (citing *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989)). Since the Examiner is relying upon multiple references to make the rejection under 35 U.S.C. §102(b), the rejection is improper and should be raised under 35 U.S.C. §103(a). Applicant accordingly addresses the Examiner's rejection as properly falling under 35 U.S.C. §103(a).

Nilsson discloses oligonucleotide probes having two target-complementary segments connected by a linker sequence. Upon recognizing a specific nucleic acid molecule, the ends of the probes are joined by ligase to form circular DNA molecules which are catenated to the target sequence within the nucleic acid molecule (*see e.g.*, abstract). The reference also discloses a buffer solution comprising 10mM tris (pH 7.5), 10 mM Mg(Ac)₂, 50 mM KAc, 10mM ATP) used to wash the hybridized oligonucleotide probes prior to treatment with ligase (*see e.g.*, Fig. 4 legend).

Rajagopalan teaches aqueous buffer solutions suitable as pharmaceutically acceptable carriers comprising mixtures of tris, phosphate, citrate, or bicarbonate, sterile water, physiological saline, and balanced ionic solutions. Column 7, lines 55-68.

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Shelley discloses pharmaceutical carrier systems comprising mixtures of acetaminophen, polyethylene glycol, propylene glycol, water, polyvinylpyrrolidone and alkali metal acetates for enhancing the solubility of acetaminophen for encapsulation in soft gels. Column 2, lines 45-57 and 52-64.

Mills teaches pharmaceutical compositions comprising mixtures of urea, alkaline potassium salts, and magnesium salts for treating equine diseases. Column 4, lines 37-40.

Rapaport discloses pharmaceutical preparations comprising mixtures of AMP, ADP, ATP, adenosine, and phosphate salts in isotonic solutions for promoting increases in liver, red blood cell, and blood plasma ATP pools in small mammals. Column 5, lines 27-38.

In contrast to the teachings of the cited art, the present invention is directed to compositions for targeting double stranded nucleic acids comprising pharmaceutically acceptable carriers and an effective amount of a padlock probe oligonucleotide.

To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. In re Vaack, 947 F2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The Examiner states that the solution (*i.e.*, 10mM tris (pH 7.5), 10 mM Mg(Ac)₂, 50 mM KAc, 10mM ATP) disclosed in *Nilsson* comprises a pharmaceutically acceptable carrier, as claimed in the invention, in view of the teachings of *Rajagopalan*, *Shelley*, *Mills*, and *Rapaport*.

Applicant respectfully traverses.

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There is no suggestion or motivation in the cited references to selectively combine the teachings of the secondary references with *Nilsson* to make a composition comprising a pharmaceutically acceptable carrier and an effective amount of a padlock probe oligonucleotide. In particular, there is no suggestion or motivation in the references for selecting and combining one component from each of the aqueous solutions disclosed in the secondary references. In particular, the Examiner relies on *Rajagopalan* to provide the tris component in *Nilsson*. (e.g., tris from the group tris, phosphate, citrate, or bicarbonate, sterile water, physiological saline, and balanced ionic solutions). Another component from *Nilsson*, potassium acetate, is derived from the acetaminophen containing solutions disclosed in *Shelley* (e.g., alkali metal acetates from polyethylene glycol, propylene glycol, water, polyvinylpyrrolidone and alkali metal acetates). Yet another component in *Nilsson*, magnesium acetate, is derived from the solutions disclosed by *Mills* for treating equine diseases (e.g., magnesium salts rather than magnesium acetate from urea, alkaline potassium salts, and magnesium salts). The final component of *Nilsson*'s buffer, ATP, is derived from the solutions disclosed in *Rapaport* for promoting increases in ATP pools (e.g., ATP from the group AMP, ADP, ATP, adenosine, and phosphate salts in isotonic solutions). The Examiner relies on these references in combination to reach the solution described in *Nilsson* (i.e., 10mM tris (pH 7.5), 10 mM Mg(Ac)₂, 50 mM KAc, 10mM ATP). This purportedly demonstrates the *Nilsson* solution as being operative as a pharmaceutically acceptable carrier for use with an effective amount of padlock probe oligonucleotide. However, nothing in the cited references teaches that the padlock oligonucleotide probe and *Nilsson* solution should be combined or that the particular components from each secondary reference should be chosen in combination to produce the *Nilsson* solution. Accordingly, the claims are neither anticipated nor obvious over the art of record.

Claim Rejections – 35 U.S.C. §112, First Paragraph

Claim 11 (and dependent claim 12) stand rejected under 35 U.S.C. §112, first paragraph as containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the invention at the time the application was filed. More particularly, the Examiner argues that the specification provides no examples of the “mutually reactive compounds” which are recited in claim 11.

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The chemical compounds and functional groups which are capable of joining the end parts of oligonucleotides are well recognized in the art. For Example, *Gryaznov, et al. Nucleic Acids Res*, 22(12):2366-9 (1994) describes the increased selectivity of interactions between oligonucleotide probes and target nucleic acids due to the inclusion of thiophosphoryl and bromoacetamido groups at the respective ends of each oligonucleotide probe, wherein efficient autoligation takes place when the oligonucleotide probes hybridize in a continuous mode to the same complementary strand such that a thiophosphoryl group on one strand and a bromoacetamido group on another strand are brought together into close proximity. Applicants have enclosed a copy of the *Gryaznov* reference for review by the Examiner.

Applicants accordingly respectfully request withdrawal of the rejection under 35 U.S.C. §112, first paragraph of amended claim 11 (and dependent claim 12).

Claim Rejections – 35 U.S.C. §112, Second Paragraph

Claim 11 (and claim 12 which depends therefrom) stands rejected under 35 U.S.C. §112, second paragraph as being indefinite. These claims are also rejected under §112, first paragraph as being insufficient written description. Both rejections relate to the term “mutually reactive compounds” recited in the claims. Applicant reiterates that chemical compounds and functional groups which are capable of joining the end parts of oligonucleotides are well recognized in the art. Applicant has enclosed a copy of the *Gryaznov* reference for the Examiner’s review. Accordingly, Applicant respectfully requests withdrawal of the rejection under 35 U.S.C. §112, second paragraph of amended claim 11 (and amended claim 12 which depends therefrom).

Claim 12 stands rejected under 35 U.S.C. §112, second paragraph for failing to particularly point out and distinctly claim the subject matter of the invention. Applicants have amended claim 12 to correct errors in claim dependency. Applicant accordingly, respectfully requests withdrawal of the rejection under 35 U.S.C. §112, second paragraph of amended claim 11 (and amended claim 12 which depends therefrom).

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CONCLUSION

Applicant submits that the amended claims are now in condition for allowance and an notification of such is solicited.

Respectfully submitted,

Dorsey & Whitney LLP

Date:

7/8/03



Richard F. Trecartin, Reg. No. 31,801
Filed under 37 C.F.R. §1.34(a)

Four Embarcadero Center, Suite 3400
San Francisco, California 94111-4187
Telephone: (415) 781-1989

1111604

Enhancement of selectivity in recognition of nucleic acids via chemical autoligation

Sergei M. Gryaznov*, Ronald Schultz¹, Surendra K. Chaturvedi¹ and Robert L. Letsinger¹
 Lynx Therapeutics Inc., 3832 Bay Center Place, Hayward, CA 94545 and ¹Department of Chemistry, Northwestern University, Evanston, IL 60208, USA

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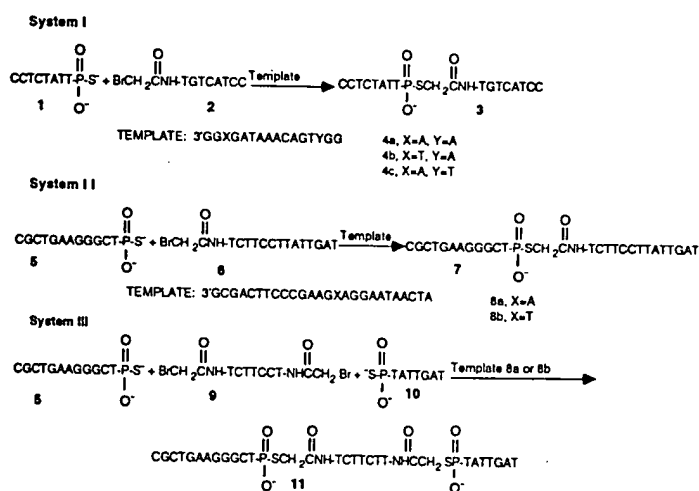
ABSTRACT

A new approach to increase the selectivity of interaction between oligonucleotide probes and target nucleic acids is described. In place of a single, relatively long oligonucleotide probe, two or three short oligomers terminated by thiophosphoryl and bromoacetamido groups are employed. Fast and efficient autoligation takes place when the oligomers hybridize in a contiguous mode to the same complementary strand such that a thiophosphoryl group on one strand and a bromoacetamido group on another are brought into proximity. A single nucleotide mismatch for the short probes leads to marked reduction in the rate of autoligation. The binding affinity of the product is close to that for a natural probe of the same length. This approach could have potential in oligonucleotide-based diagnostics, chemical amplification systems, and therapeutic applications.

INTRODUCTION

Synthetic oligonucleotides are being extensively used as sequence-specific antisense agents (1-3), as well as probes for hybridization based detection assays of nucleic acids (4, 5). One of the problems remaining to be resolved, and especially relevant to antisense applications, concerns the formation of complexes with targets that are not fully complementary. Relatively long oligomers (e.g. ~20-mers) are needed to provide a unique sequence and a high binding affinity in targeting human genetic segments; however, the longer oligomers are not highly selective as antisense agents (6). Short oligomers (e.g. 7-12 mers), which bind with low affinity, are much more effective in discriminating between complementary strands and targets containing one or more mismatches. We describe here an approach designed to capitalize on the positive features of both the longer and the shorter oligomeric agents (7). It is based on *in situ* chemical ligation of relatively short oligonucleotide fragments bearing electrophilic bromoacetamido and nucleophilic phosphorothioate monoester groups at their termini. As previously reported, rapid and efficient autoligation (ligation without condensing agents) takes place when such oligomers hybridize in a contiguous mode to a complementary strand (8).

In testing the effects of mismatches in target sequences on autoligation of relatively short oligonucleotides, we have examined the efficiency of coupling in the following three systems.



EXPERIMENTAL SECTION

General methods

Ion exchange high performance liquid chromatography (IE HPLC) was carried out on a Dionex Omni Pak NA 100 4×250 mm column at pH 12 (10 mM NaOH) with a 2%/min gradient of 1.5 M NaCl in 10 mM NaOH; 1 mL/min flow rate. For analyses in System I, the gradient solution also contained MeCN (2% by volume). Reversed phase (RP) HPLC was carried out with a Hewlett Packard Hypersil ODS 5 μm, 4.6×200 mm column at pH 7 in aq. 30 mM Et₃N/HOAc with a 1%/min gradient of MeCN. Polyacrylamide gel electrophoresis (PAGE) was carried out with denaturing cross-linked 20% polyacrylamide gels (5% bis-acrylamide); R_m values are relative to xylene cyanol. Except where otherwise noted, melting curves were obtained using solutions 0.10 M in NaCl. 5 μM of each oligonucleotide, pH 7.0, by following changes in absorbance at

*To whom correspondence should be addressed

260 nm while ramping the temperature at a rate of 1°C/min. Oligonucleotides were prepared via automated synthesis using standard cyanoethyl phosphoramidite chemistry. They were isolated with the DMT group intact, and, following detritylation (80% aq. HOAc, 15 min), were purified by successive RP HPLC and IE HPLC.

Preparation of CCTCTATT-P(O)(OH)S-

This oligonucleotide 3'-phosphorothioate was prepared by the procedure described in reference 9. It was also prepared conveniently starting with '3'-Phosphate CPG' supplied by Glen Research [DMTO(CH₂)₂SO₂(CH₂)₂O-succinyl-lcaa-CPG]. In the latter case the DMT group was removed by DCA treatment and the support was phosphitlated with a DMT-thymidine cyanoethylphosphoramidite reagent. Following sulfurization with a 5% solution of S₈ in CS₂/pyridine (1/1 v/v) (45 min reaction) the oligonucleotide was synthesized by standard protocol. After isolation by preparative HPLC, the product eluted as a clean peak on RP HPLC (16.7 min) and on IE HPLC, pH 12 (16.4 min).

Table 1. Properties of oligonucleotides

Oligomers	R min ^a	Rm ^b	Tm °C ^c	Tm °C ^d
5	22.4	1.54	54.3	54.3
6	21.7	1.32	40.8	31.8
7	26.4	0.83	65.3	59.0
9	16.6	1.70	18.3	<5
10	18.4	1.86	17.0	17.0
11	26.5	0.81	63.0	52.2 ^e
12 ^f	26.3	0.85	66.8	60.0

^aRetention time on IE HPLC, pH 12.

^bPAGE, mobility relative to xylene cyanol.

^cTm for duplex with complementary template 8a in 20 mM MES and 20 mM MgCl₂, 1 μM in each oligomer.

^dTm for duplex with mismatched template 8b under same conditions as in footnote c.

^eVery low hyperchromicity (~7%) observed for this curve.

^fOligomer 12 is a natural phosphodiester compound possessing the same nucleotide sequence as 7 and 11.

It formed a complex with template 4a exhibiting Tm 23°C (0.2 M NaCl); interaction with mismatched template, 4b, under the same conditions was very weak (Tm <2°C). In support of the presence of a terminal thiophosphoryl group, oxidation of 1 A260 unit of the oligomer in 10 μL of water with 1 μL of 1 M aq. K₃Fe(CN)₆ (3 hr at 4°C) afforded a product eluting later on IE HPLC, as expected for oxidation of a terminal oligonucleotide phosphorothioate to a dimeric disulfide derivative (9).

Preparation of BrCH₂C(O)NH-TGTCATCC

To 5 A₂₆₀ units of NH₂-TGTCATCC (prepared as in ref. 10; elution time 16.5 min for IE HPLC at pH 12; Tm = 32°C with template 4a, Tm = 15°C with mismatched template 4b) in 15 μL of 0.2 M sodium borate buffer, pH 8.5, at room temperature was added 15 μL of 0.4 M N-succinimidyl bromoacetate in MeCN. After 30 min the mixture was diluted to 0.5 mL with water and desalted on a NAP-5 column. The bromoacetamido derivative (3.5 A₂₆₀ units) was isolated by RP HPLC followed by lyophilization. It was homogeneous (~99% as a single peak) as judged by RP HPLC and by IE HPLC at pH 7 (30 mM Et₃N/HOAc, 10% MeCN with a 2%/min gradient of 1.0 M NaCl, 30 mM Et₃N/HOAc, 10% MeCN). When analyzed by IE HPLC at pH 12, however, two peaks appeared—a major peak (~75%) at 15.4 min and a minor one (~25%) at 14.5 min. Rechromatography of the product collected from the major peak again yielded the two peaks in about the same ratio. We conclude that the minor peak is a degradation product (probably the hydroxyacetamido derivative) formed on exposing the bromoacetamido derivative to the strongly alkaline solution (pH 12) used in the chromatography. In agreement with this conclusion, the percentage of material in the faster eluting material increased to ~60% and then to >90% when the bromoacetyl derivative was exposed to a solution at pH 12 for 1 hr and for 2.7 hr, respectively, before chromatography.

Compounds for Systems II and III

These compounds were prepared as described for the oligomers in System I and in reference 8–10. Some physico-chemical

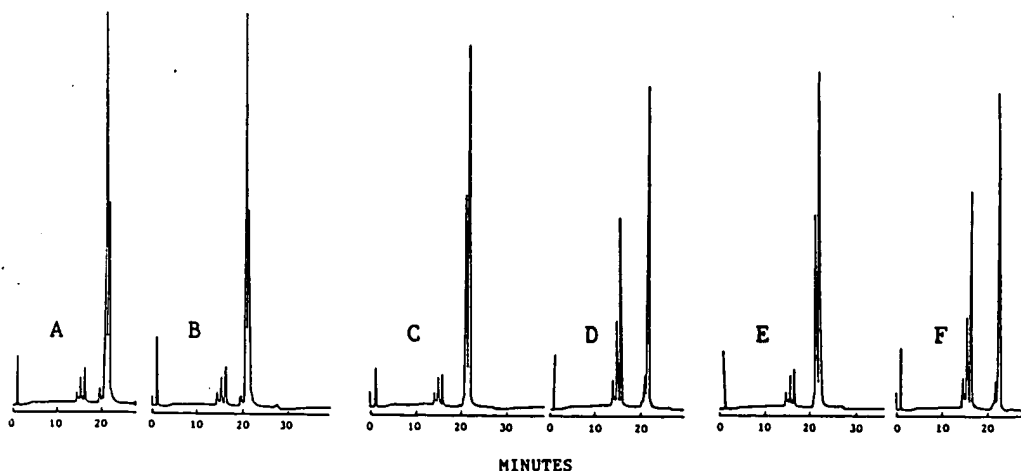


Figure 1. IE HPLC of products from System I (1 + 2 + template 4a, 4b, or 4c) in buffer I, 5 min reaction time: A, with 4a at 0°C; B, with 4a at 30°C; C, with 4b at 0°C; D, with 4b at 30°C; E, with 4c at 0°C; F, with 4c at 30°C. The peaks and assignments for A and B are, respectively: ~15.3 and 14.5 min (bromoacetamido derivative 2 and its hydrolysis product formed during analysis); 16.5 min (phosphorothioate 1); 20.9 min (template 4a); 21.5 min (product 3). The assignments are the same for figures B–F except templates 4a and 4b elute at ~22.5 min, after the product peak. The recorder sensitivity was 0.5 for A, B and 0.1 for C–F.

characteristics of the oligonucleotides are summarized in Table 1. Oligomer 6 proved to be considerably more stable than oligomer 2 in solution at pH 12. On direct IE HPLC at pH 12 only about 3% was hydrolyzed, and after standing in a pH 12 solution for 4 hr, chromatography at pH 12 showed only about 12% conversion to the faster eluting product. The hydrolysis rate of the bromoacetyl derivative is therefore related to the oligonucleotide sequence, and in the case of oligomer 2 may be enhanced by the proximity of a guanine residue.

Ligation experiments

Ligations were carried out with 1 μ M solutions of each oligomer in an aqueous solution (buffer I: 15 mM sodium phosphate and 0.2 M NaCl, pH 7.0; or buffer II: 20 mM MES and 20 mM MgCl₂, pH 7.05; or buffer III: 0.1 M NaCl). At the end of the designated time an aliquot was injected in the HPLC unit for IE chromatography at pH 12. This pH was selected to denature oligonucleotide complexes in the system. The structure assigned

to ligation product 3 is supported by the elution time on IE HPLC (close to values for 16-mers 4a–c) and the melting curve for the complex of 3 with 4a; $T_m = 56^\circ\text{C}$ for 4 μ M of the complex in 0.1 M NaCl. Under the same conditions $T_m = 62^\circ\text{C}$ for a natural phosphodiester duplex with the same nucleotide sequence. In addition to the elution time on IE HPLC, mobility on gel electrophoresis, and T_m value for thermal dissociation of the complex formed with a complementary oligonucleotide (see Table 1), the structures assigned to compounds 7 and 11 were supported by selective cleavage of the $-\text{NHCOCH}_2\text{SPO}_2^-$ links with NaI₃ by the procedure described in reference 8.

RESULTS AND DISCUSSION

Ligation studies with CCTCTATT-OP(O)(OH)S⁻ (1) and BrCH₂C(O)NH-TGTCATCC (2) (System I) were carried out in solutions 1 μ M in each oligomer at pH 7.0 (15 mM sodium phosphate and 0.2 M NaCl). Products were analyzed by ion

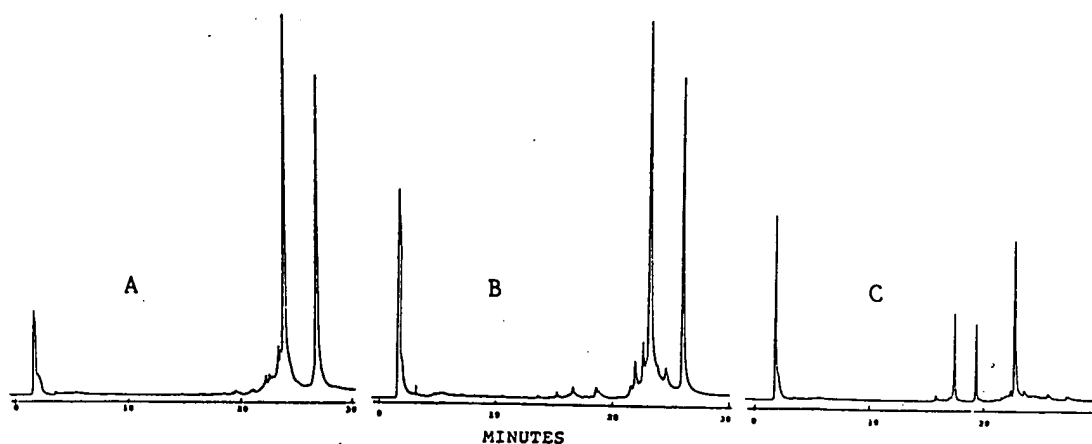


Figure 2. IE HPLC of products formed in 5 min in buffer II at 0°C for: A, Systems II (5 + 6 + template 8a); B, System III (5 + 9 + 10 + template 8a); C, System III without any template (5 + 9 + 10). The peaks at ~23 and 25 min correspond to template 3a and ligation product 7 or 11, respectively; the peaks at ~17, 18, and 22 min correspond to oligomers 10, 9, and 5.

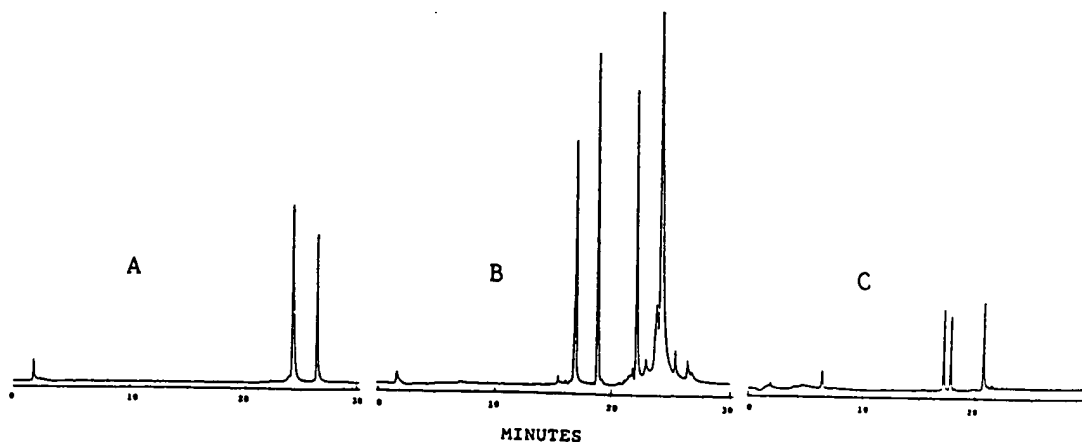


Figure 3. IE HPLC of products formed after 2 hr in buffer III at 23°C for: A, System III with fully complementary template 8a; B, System III with mismatched template 8b; C, Products of cleavage of 11 with NaI₃ followed by DTT according to reference 9; note that 11, which elutes at ~26.5 min, was transformed to three oligonucleotide cleavage products, eluting at ~17, 18, and 20 min.

exchange HPLC at pH 12. In absence of a template, no ligation was observed for reactions carried out for 45 minutes at either 0°C or 30°C. In marked contrast, rapid ligation occurred when an appropriate template was present. HPLC profiles are shown in Figure 1 for reactions carried out for 5 minutes at 0°C or 30°C in presence of a template (~10% excess template) that was either fully complementary or contained one mismatch. The tallest peak in each profile corresponds to the template. The ligation product is the peak eluting immediately after the template (compound **4a**) in Figure 1A,B and just before the template (compound **4b** or **4c**) in Figure 1C,E. Residual thioate (**1**) appears at 16.5 minutes and the residual bromoacetamido derivative appears as a double peak at 14.5 and 15.3 minutes (the 14.5 peak is formed during chromatography at pH 12; see experimental section). At 30°C the ligation efficiency is markedly dependent on proper base pairing. From the peak areas we estimate that the conversion of **1** and **2** to **3** was approximately 75% when the fully matched template (**4a**) was present (Figure 1B), but ligation amounted to less than 5% when a single mismatch occurred in a segment of the template binding to either oligomer **1** (Figure 1D) or to oligomer **2** (Figure 1F). Proper base pairing proved to be much less important at 0°C, where the binding affinity is much greater even when the template has a mismatched base. At this temperature, conversion to the ligated product amounted to 85%, 78%, and 74% for mixtures containing **4a** (matched template, Figure 1A) and **4b**, and **4c** (mismatched templates, Figure 1C,E), respectively (11).

These results are supported and extended by work with another sequence involving double (System III) as well as single ligation (System II) of the modified oligonucleotides. After 5 minutes of ligation in buffer II (20 mM MES containing 20 mM MgCl₂) at 0°C, yields of the 26-mer products of ligation from System II (**7**) and System III (**11**) were ~97% (Figure 2A,B). Practically no ligation was observed in System III when the template was absent (Figure 2C). Ligation on mismatched template **8b** at 0°C produced **7** and **11** in yields of 97% and 67% respectively, probably as a consequence of formation of partially complementary complexes. Better discrimination was achieved in low salt buffer III (0.1 M NaCl) and at a higher annealing temperature. Only 18% of ligation product **7** was observed in System II after 2 hours of reaction with mismatched template **8b** at 37°C; at 41°C the yield was only 7%. Under the same conditions but with fully complementary template **8a** the yield was ~98% at both temperatures.

In the four component system (III) at 23°C, only ~5% of product **11** was detected after 2 hours incubation with mismatched target **8b**, in contrast to 94% for the fully complementary template, **8a** (Figure 3A,B). These data indicate that formation of a duplex is important for an efficient ligation reaction. In Systems II and III a very small fraction of oligomers are in the duplex state with mismatched template **8b** at 41°C and 23°C (T_m's 31.8°C and <5°C; Table 1); therefore low yields of ligation products are obtained. In contrast, a substantial fraction of molecules hybridize with fully complementary template **8a** under analogous conditions (T_m's 40.8°C and 18.3°C; Table 1), resulting in formation of ligation products **7** and **11**.

In summary, this approach provides an effective means for discrimination of duplexes containing even a single mismatched base pair and could potentially increase the specificity of oligonucleotide ligation amplification techniques (12) or antisense applications.

ACKNOWLEDGEMENTS

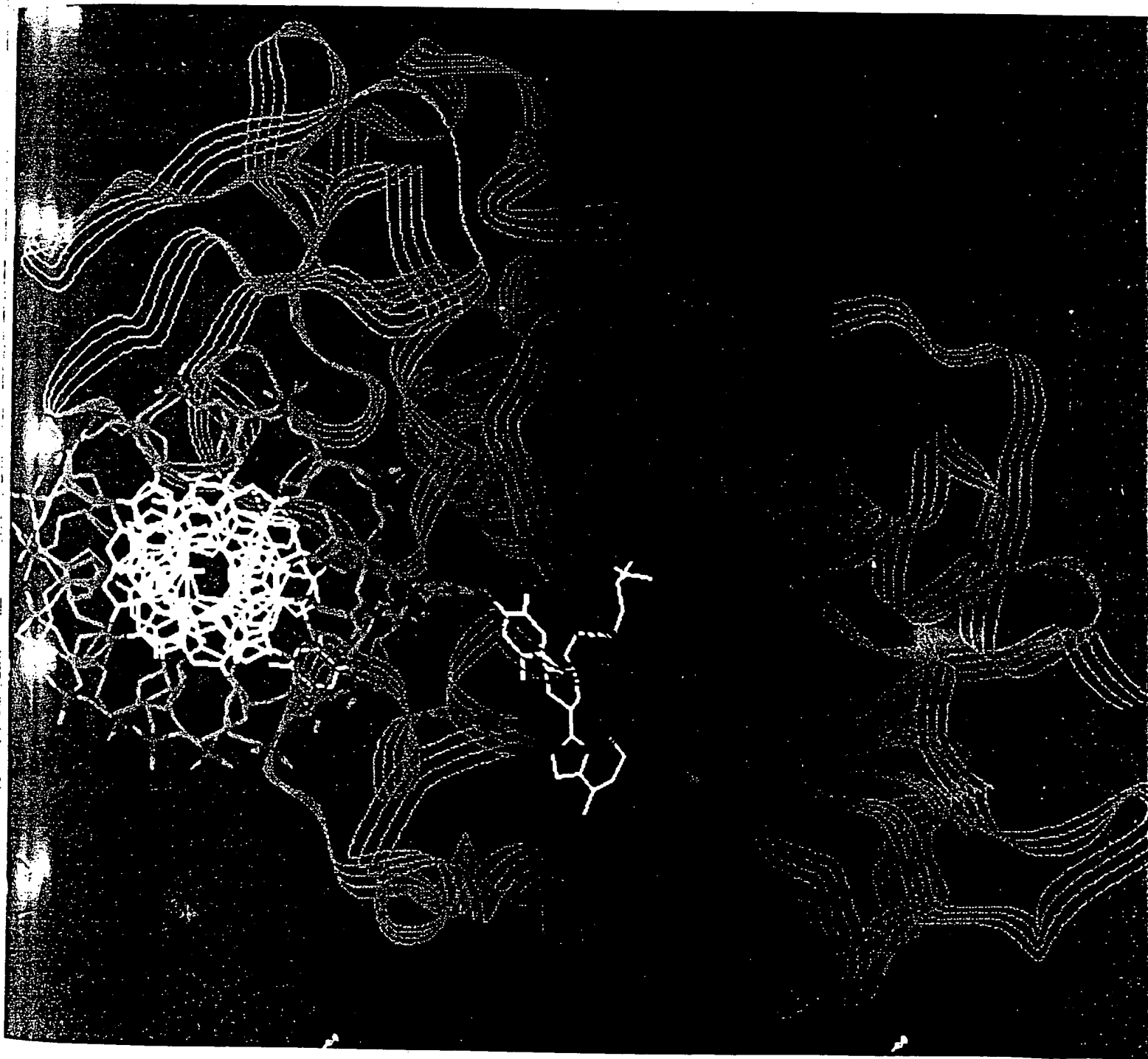
The work at Northwestern University was supported by research grants from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases (Grant UOI AI24846) and the National Institute of General Medical Sciences (Grant 10265).

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